THE RESIDUAL ANTIBACTERIAL EFFECTS OF RADIOPAQUE DOUBLE ANTIBIOTIC PASTE AFTER VARIOUS TREATMENT TIMES

by

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Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, June 2018.

Thesis accepted by the faculty of the Department of Endodontics, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

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ACKNOWLEDGMENTS

I would first like to thank my parents, Rick and Pam Biggerstaff, for providing endless support and love over the last 31 years of my life. I owe them more than I can give for being beside me along this long journey and helping to shape me into the person I am today. They are shining examples of where hard work gets you, and they have instilled this into me. Without their dedication to me, I have no idea where or who I would be.

I want to thank Dr. Spolnik for accepting me into this program and affording me the opportunity of a lifetime. Without hesitation, Dr. Spolnik is willing to share his wealth of knowledge and experience, which is truly invaluable. I have the utmost respect for everything he does for all the residents and the entire department. I have no idea how he manages so many life avenues at such an exceptional level. I can only hope one day, I will be able to have half the success and confidence Dr. Spolnik possesses. For years to come, I know I will mentally have Dr. Spolnik over my shoulder ensuring I am performing at the best of my abilities.

Over the last two years, Dr. Bringas has become not only a phenomenal mentor, but a friend. Every week, I would look forward to Dr. Bringas staffing the VA because I knew I was going to learn something more about root canals while also discovering something new unrelated to endodontics, whether it was about novel technology, sports, cars, or a show to watch. Dr. Bringas has taught me to be pragmatic in making decisions

by backing treatment choices with evidence, while also being able to think on my feet to improvise in difficult situations. I truly look forward to more conversations and time with Dr. Bringas in the future.

I want to thank Dr. Ehrlich for his dedication to the program and help in getting this project rolling. Last year he took the job as our classic literature professor, and I could not have been happier. I was able to see first-hand the extent of knowledge Dr. Ehrlich possesses, and he did a fantastic job imparting it. He is not only very intelligent, but also does an unbelievable amount for his family. I commend him for everything he does for them while also providing us with a great education.

Dr. Warner is always walking in with a smile that is contagious. He has taught me about life outside of endodontics, which I sincerely value. I do not understand how he is able to handle the undergraduate program and all the students with their complaints, but it certainly is not an easy task and needs to be recognized.

I want to thank Ghaeth Yassen for everything he has done for us during this long journey of research. He was the one who continued to keep us on track and ensured we worked through our projects in a timely manner. Even from a location in another state, he still takes time out of his busy life to help us to improve our projects and presentations. Without Ghaeth, I would have been lost in attempting this project and cannot thank him enough for all the time he spent in refining and improving our research.

Dr. Gregory has also always been there to help us when in trouble and his aid needs to be acknowledged. He has been very patient with us in wrapping up this project and never showed any signs of frustration toward us, even when we maybe deserved it for some procrastination. This school is extremely fortunate to have someone like Dr.

Gregory helping to educate the students and providing research assistance whenever asked.

Lastly, I want to thank my co-residents for making this residency an unforgettable experience. It has been a beautiful thing to have people around you experiencing the same struggles, when we are constantly pushing each other to improve. We have grown to be a family and I could not imagine going through this program with anyone else.

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INTRODUCTION

Immature teeth that exhibit pulpal necrosis have been, and continue to be, a challenge for endodontists to treat. For successful regenerative therapy, a disinfection protocol is paramount (1). After disinfection, purposefully initiating bleeding of the periapical tissue is imperative, as it allows undifferentiated stem cells to accumulate in the canal space and initiate the host response (2). To further promote disinfection and repair, the root canal system can be medicated with a triple antibiotic paste (TAP) comprised of metronidazole, ciprofloxacin, and minocycline for one to four weeks (3). Once the pulp space has been disinfected, a hand file is used to lacerate the periapical tissue, which causes hemorrhage. This blood and clot formation acts as a scaffold, allowing stem cells to form pulp-like tissue, often comprised of cementum, bone, and PDL (4,5). This process is known as regenerative endodontics, which has the potential to cause tissue to proliferate into the canal space of an immature tooth with pulpal necrosis (6).

As the name suggests, immature teeth are not fully formed; they lack root wall thickness and have a relatively small crown to root ratio. These characteristics increase the risk for cervical root fracture in immature teeth with necrotic pulps (7,8). Apexification has been the treatment of choice in immature teeth with necrotic pulps due to the fact that an open apex makes the obturation step of traditional endodontics ineffective. Both MTA and calcium hydroxide have been used to treat immature teeth in order to create an apical barrier to contain the obturation material within the canal space (9-11); although the issue of the compromised crown to root ratio remains, as does the risk for cervical root fractures.

The solution may be regenerative endodontics, which has been documented as allowing both the root length and thickness to continue developing in immature teeth (12-14). Induced bleeding into the canal from the periapical area of the immature tooth with a necrotic pulp creates a scaffold in the form of a blood clot, while also delivering the mesenchymal stem cells from the apical papilla into the root canal system (2). Additionally, growth factors such as PDGF, VEGF, EGF, and FGF2 [15,16] are derived from dentin or platelets. Thus, all three components necessary for tissue engineering are incorporated into regenerative endodontics: stem cells, scaffold, and growth factors (17). However, unless the root canal is disinfected, no tissue engineering can take place. Disinfection technique options currently include sodium hypochlorite, calcium hydroxide, TAP and double antibiotic paste (DAP). Research is being conducted to determine which antimicrobial option is likely to result in successful regenerative endodontic therapy.

Sodium hypochlorite has been widely used in endodontics primarily due to its strong disinfectant ability and its effective dissolution of organic tissue (18). Because of the toxicity of full strength sodium hypochlorite, it could decrease stem cell viability as well as affect stem cell attachment (19,20). Considering this cytotoxicity, it is suggested to utilize lower concentrations at the initial disinfection appointment and to exclude it at the subsequent appointments in order to achieve stem cell proliferation and attachment (21).

Calcium hydroxide has also been to be a very useful disinfectant and thus has become a standard in endodontics. It has been shown that calcium hydroxide provides an alkaline environment that allows for LPS hydrolysis (22-25). Calcium hydroxide does

come with its drawbacks. Studies have demonstrated that it can lead to reduced fracture strength after thirty days (26), lowered dentinal flexural strength (27), and collagen degradation (28). Due to some of these disadvantages, antibiotic pastes may be a better alternative for canal disinfection.

Currently, research has predominantly focused on different antibiotic combinations with assorted concentrations when tackling these immature teeth with pulpal necrosis. TAP, an antibiotic mixture composed of equal parts ciprofloxacin, metronidazole, and minocycline, was discovered to be a superior combination in the disinfection of root canals (3, 29). However, TAP has some shortcomings, which include tooth discoloration, dentin demineralization, and cytotoxicity to viable stem cells (28, 30, 31). It has been suggested to lower concentrations of TAP to avoid destruction of these stem cells (21, 32)

DAP was introduced as an alternative antibiotic dressing in regenerative endodontics procedures (33). DAP is composed of ciprofloxacin and metronidazole. With the elimination of minocycline, DAP does not produce the unwanted effects of tooth discoloration (34). As with TAP, higher levels of DAP will still create a cytotoxic environment for stem cells (32). A case report demonstrated that DAP is effective in apical closure and thickening of dentinal walls within the canals of an immature tooth with a necrotic pulp (35). Nevins and Cymerman also found success when using DAP in four separate cases of immature teeth with necrotic pulps. This case series showed that DAP was effective in greatly reducing symptoms and the size of periapical radiolucent area. Additionally, this equal part ciprofloxacin to metronidazole paste was shown to be effective in tissue repair without the presence of minocycline (36).

DAP and its effect on endodontic pathogens including Enterococcus faecalis and Porphyromonas gingivalis has been investigated in comparison to TAP and calcium hydroxide. DAP was able to disinfect dentin as well as TAP without the negative effects of tooth discoloration (37). Furthermore, various dilutions of DAP were investigated for their antibacterial effect on E. faecalis. It was shown that concentrations of 0.125, 0.25, 0.5, 1, and 10 mg/mL of DAP were all an effective antimicrobial agent against E. faecalis, but 0.125 mg/mL demonstrated the lowest cytotoxicity of dental pulp stem cells (38, 39).

Several of the regenerative endodontics studies involving DAP frequently utilize E. faecalis within their protocol, but endodontic infections are likely to be more of a multi-species biofilm as opposed to a single bacterium (40). It has been shown that clinical bacterial biofilm in primary endodontic infections consists of several different bacterial phyla (41). Consequently, it would be beneficial to investigate DAP using a clinically isolated bacterial biofilm. As opposed to studying DAP solely against E. faecalis, utilizing a clinical isolate will allow for a more applicable and accurate representation of the antimicrobial properties of DAP for clinical practice.

Few studies have been conducted on the residual antibacterial effects of DAP, but they have found that non-radiopaque DAP can provide effective residual antimicrobial activity against 3 week old bacterial biofilms (42, 43). Antibiotics have their greatest effect during the reproductive cycle of bacterial cells (44), thus it is beneficial to have an extended contact time between the antibiotic paste and bacterial cells within the canal system. The residual antibiotic effect demonstrates the strong affinity of the antibiotic paste to bind to dentin and being released in an active form (45). Since regenerative

endodontic procedures do not require the canal system to be sealed or filled, there is a potential for bacterial growth within the canal following appointments. Consequently, it would be advantageous to sustain an aseptic canal system for an extended period of time (46). In concentrations of 5, 50, and 500 mg/mL, DAP has demonstrated a stronger residual antimicrobial effect for 4 weeks of dentin pretreatment when compared to 1 week of dentin pretreatment (43). This emphasizes the importance of investigating the residual antibiotic effects of DAP at different treatment times.

Antibiotic pastes used in dentistry today do not consist of radiopaque fillers. Without a radiopaque property, interpreting the quality of paste application is impractical. In dentistry, materials are often composed of various radiopaque fillers such as barium sulfate to allow for visualizing of the material on a radiograph (47). With a radiopaque DAP, the clinician will be able to ensure that the canal system has been appropriately filled, which is critical in regenerative endodontics for large, open apices of immature teeth. However, with the addition of radiocontrast salts, the antibacterial effects of antibiotic pastes may be altered resulting in reduced efficacy. Considering this, it is pertinent to perform an investigation into the antibacterial stability of radiopaque DAP.

OBJECTIVE

To investigate the residual antibacterial effect on radicular dentin pretreated for 1 or 4 weeks with various dilutions of radiopaque DAP (1, 10 and 25 mg/mL).

HYPOTHESES

Null: Tested concentrations (1 mg/mL, 10 mg/mL, and 25 mg/mL) of radiopaque DAP will have no significant residual antibacterial effects, regardless of the application time.

Alternative: Tested concentrations (1 mg/mL, 10 mg/mL and 25 mg/mL) of radiopaque DAP will have significant residual antibacterial effects, regardless of the application time.

REVIEW OF LITERATURE

HISTORY OF ENDODONTICS

Oral disease and its treatment have made great strides since Sumerian text from around 5000 B.C. described "tooth worms" to be the cause of toothaches. For centuries, dental pain was handled through removal of the source with replacement of the missing dentition (48). In the 18th century, Pierre Fouchard, thought of as the father of modern dentistry, challenged this "tooth worm" notion by associating tooth decay with sugar consumption. In Fouchard's book, *The Surgeon Dentist*, he discusses various techniques managing dental disease and pathosis that paved the way for current dental procedures. In an approach to combat dental infection, he described a technique involving the preparation of a hole into the pulp to allow for adequate drainage of the infection. Following this, the tooth is sealed with lead foil serving as a rudimentary obturation material. Fouchard further explains a process of dental pulp extirpation and utilization of cloves for the treatment of gross carious lesions (48,49). Fouchard's elementary treatment protocols represent some of the early beginnings of modern endodontics.

Following Fouchard, several others began performing various endodontic techniques aiding in the growth of the field. In 1756, nearly 30 years after the publication of *The Surgeon Dentist*, Philipp Pfaff of Germany utilized lead or gold as a pulp capping material (50). In 1766 Robert Woofendale was the first provider in the US to record and perform an endodontic procedure involving heat cauterization of the pulp with application of cotton pellets to lessen odontogenic pain (51). Almost 35 years later, Frederick Hirsch reported using percussion diagnostic tests to assess the periapical status

of teeth to localize the source of odontogenic pain. He found that teeth sensitive to percussion were the teeth exhibiting dental pain (49).

At the start of the 19th century, dental professionals started to understand the significance of pulp vitality, which brought forth the advent of "The Vitalistic Era" (48). This new era can be partially credited to Charles Bew who initially described pulpal circulation as blood flow entering the tooth through the apical foramen. In 1826 Leonard Koecker published *Principles of Dental Surgery* in which he discussed how necrosis of the pulpal tissue would cause the tooth to die. This led Koecker to support pulp capping procedures parallel to those described by Pfaff to preserve pulpal vitality (52).

Later in 1829, SS Fitch brought forth the vitalistic theory, which included his idea that the entire tooth, similar to a hollow bone, is vital (48,52). He explained how the coronal aspect of the tooth was supplied by pulpal circulation whereas the periodontal ligament as well as pulpal circulation provided vascularity to the roots of teeth. These beliefs led to the rise of decoronation procedures where the clinical crown was removed after pulpal extirpation. Opposing this philosophy was the "non-vitalistic theory" which included the school of thought that enamel and dentin lacked pulpal circulation. Without vascularity, these tissues would not possess the ability of self-repair. Supporters of this ideology believed that the removal of pulp tissue had no detrimental effect on remaining tooth structure (48).

Endodontics began to see more and more changes as time progressed including various medicaments being used for differing purposes. In 1836, Shearjashub Spooner began to utilize arsenic trioxide to devitalize pulp tissue prior to pulpectomy procedures to aid in pain relief (53). At the same time, Jacob and Joseph Linderer experimented with

using narcotic oil over pulp exposures to achieve pulpal anesthesia (54) Further advancements were continuing to be made, including the introduction of formocresol by J.P. Buckley as a fixative agent, which can still be found in some practices today (55).

In addition to the introduction of new medicaments, novel techniques for the obturation of the root canal space began to develop. In 1809 Edward Hudson used gold foil to fill root canal space (53). Baker went on further to demonstrate cleaning of the root canal space prior to filling the canals with gold foil. He described this in the *American Journal of Dental Science* in 1839, which laid the foundation for canal debridement and subsequent obturation (48). During this period, dental professionals were also finding other methods to obturate root canals including using beechwood plugs saturated with creosote (56). In the 1860s, gutta percha as a sole obturation material came into light with G.A. Bowman advocating its ability to fill root canals while E.L. Clarke began filling roots with heated base-plate gutta percha (48,56). Later, G.A. Bowman began experimenting with other techniques and found that utilizing chloroform to soften the gutta percha cone allowed for a better apical 3-dimensional fill, which was termed chlorapercha (53).

Many modern instruments used by endodontists today are based on instruments developed in the 1800s. Edwin Maynard was a pioneer in creating novel instruments including the invention of the broach in 1838, which he had fashioned from the twisting of a metal watch spring. Sanford Barnum demonstrated the use of a rubber dam during restorative work for isolation needs in 1864. Barnum’s ingenuity in developing rubber dams would later support the standard of care when performing root canals due to its ability to avoid saliva contamination while providing an aseptic environment (56).

Dental practitioners began to value the importance of an aseptic environment, as the impact of microorganisms on subsequent pulpal and periapical disease became better understood. In 1878 G.O. Rodgers advocated that pathogens might be the major cause of pulpal pathosis. With this belief, adequate removal of the microbial insult could lead to better success in eliminating pulpal disease (57). This theory of asepsis began to gain momentum amongst the dental community. Others including Arthur Underwood began supporting the elimination of microorganisms from the root canal system. Underwood was recorded promoting the use of antiseptics during root canal procedures to remove any microbial insult and treat suppuration of the pulp (48).

Substantial innovations during the early 1900s shaped the way dentistry is practiced today. In 1905, procaine (Novocaine) was first created, which gave dental practitioners a more adequate and effective anesthetic agent when compared with the formerly used cocaine. Clinicians began expanding their knowledge of anesthesia and creating more effective anesthetic techniques such as block anesthesia (58,59). Further advancements that play a major role in endodontics began to follow including the discovery and application of dental x-rays for treatment and diagnosis. Dental x-ray units began to be sold commercially in 1919 when focused x-ray beams were deemed possible with the invention of the Coolidge tube (60). This immediately led to the identification and diagnosis of not only carious lesions but also periapical disease. With this advancement, the relation between pulpal and periapical disease became clearer to endodontists (61). With the emergence of dental radiography, endodontic procedures began to become more technically advanced through the use of pre-operative and working radiographs (55). Dental radiographs expanded the boundaries of endodontics in

terms of diagnosis and treatment by allowing for a better comprehension of specific root canal anatomy and disease with a greater ability to shape the root canal system allowing for proper sealing of the root canal space.

During this same period of dental innovations, the role of endodontics in particular began to be doubted. In 1909 E.C. Rosenow and Frank Billings spawned what is known as the "Focal Theory of Infection." This idea promoted the fact that pathogenic microbes causing odontogenic infection can spread further into even distant bodily tissues leading to numerous types of systemic disorders (62). The following year, William Hunter further advocated this "focal infection theory" through an attack on dentistry. Hunter claimed, "gold fillings, gold caps, gold bridges, gold crowns, fixed dentures, built in, on, and around diseased teeth, form a veritable mausoleum of gold over a mass of sepsis to which there is no parallel in the whole realm of medicine or surgery" (63). This led to a wave of physicians beginning to recommend the extraction of all endodontically treated teeth and non-vital teeth, while some even suggested the removal of all teeth for prevention of various systemic diseases (62). For nearly 25 years, this philosophy held strong ground in the health field. Following the decline of the focal infection theory, "the scientific era" emerged, which was accompanied by further improvements in the field of dentistry (60).

At the start of the 1920s, Hermann demonstrated the use of calcium hydroxide to fill root canals. He continued to explore the capabilities of calcium hydroxide and began utilizing it for pulp capping procedures, pulpotomies, and for the treatment of necrotic cases. Hermann noted that commonly used intracanal medicaments contained toxic products that would in turn be taken up by surrounding bodily tissues. Due to this, he

pushed for the use of a more biocompatible substitute such as calcium hydroxide, which also demonstrated dentin bridge formation (60).

During the same era, obturation methods began changing and improving. In 1925 U.G. Rickert was the first clinician to utilize a sealer-coated gutta percha cone for filling root canals. Months later, Lentulo developed a rotary instrument allowing for the application of sealer within the pulp canal system as well as various pastes such as calcium hydroxide. As professionals began experimenting further with obturation methods, instruments were developed to allow for lateral condensation of gutta percha points (60).

With the introduction of penicillin to the medical community, clinicians began treating dental infections with penicillin. In 1944, Fred Adams demonstrated its use within the root canal system (60). Louis Grossman later utilized a non-aqueous carrier to apply penicillin directly into canals laden with bacterial debris. He was also recorded using paper points that had been saturated with the antibiotic to help disinfect infected root canals (53). This was a major turning point in the field of endodontics because practitioners began to value the significance of chemical disinfection. Prior to this, the community followed the notion that mechanical debridement alone would lead to an aseptic environment within the root canal space. Due to the work from professionals like Adams and Grossman, the disinfection protocol began to include both chemical and mechanical debridement (64).

With endodontics becoming a growing field in dentistry, the American Association of Endodontists (AAE) was created in 1943 with the American Board of Endodontics (ABE) following with its establishment in 1956 (65). These organizations

worked diligently to show the nation the importance of endodontics as a specific field. Finally in 1963, the American Dental Association (ADA) recognized endodontics as a dental specialty. Two years following this recognition, the first examination and certification of diplomats occurred (60).

THEORY OF ENDODONTICS

One of the most valuable studies that has molded the way endodontics is practiced today is one published by Kakehashi, Stanley, and Fitzgerald in 1965.This study is world renowned for laying the building blocks on how endodontic disease is studied and treated. The study demonstrated that pulps of germ-free rats would remain vital after being left open to the oral cavity. In sterile conditions, they found that food impaction or exposure to the oral environment would not produce pulpal necrosis and subsequent periapical disease. However, it was found that in conventional rats, the pulps became necrotic and resulted in periapical disease (66). With the publication of this study, clinicians were able to understand the importance and association between pathogenic microbes and endodontic disease.

This ideology began taking over the endodontic community. The primary objective for endodontic treatment became the removal of microorganisms and their toxins, which in turn will eliminate the microbial insult causing pulpal and periapical disease. To accomplish this, clinicians started utilizing chemical and mechanical debridement techniques with intracanal medicaments as well as novel endodontic instruments (67, 68). Periapical disease results from pathogenic microbes within the root canal space that increase in number and make their way out the apical foramen and lateral canals (69). When pathogenic bacteria are unable to be eradicated, apical periodontitis

will ensue with local inflammation and destruction of periapical tissues (70). Therefore, the success of root canal therapy can directly be associated to the reduction of endodontic pathogens (66, 71).

Three major aspects of successful endodontic therapy were described by Stewart in 1955 and are as follows: chemomechanical preparation, microbial eradication, and obturation of the root canal (72). Stewart alongside Grossman advocated the chemomechanical preparation phase as the most critical step in endodontic treatment due to it reducing the number of pathogenic microorganisms within the canal space. Grossman went on to recognize the following 13 aspects of effective endodontic therapy:

1. Aseptic technique.
2. Instruments should remain within the root canal.
3. Instruments should never be forced apically.
4. Canal space must be enlarged from its original size.
5. Root canal system should be continuously irrigated with an antiseptic.
6. Solutions should remain within the canal space.
7. Fistulas do not require special treatment.
8. A negative culture should be obtained before obturation of the root canal.
9. A hermetic seal of the root canal system should be obtained.
10. Obturation material should not be irritating to the periapical tissues.
11. If an acute alveolar abscess is present, adequate drainage must be established.
12. Injections into infectious areas should be avoided.
13. Apical surgery may be required to promote healing of the pulpless tooth.

In 1967, Schilder widened the view of successful endodontic therapy within the endodontic community. Along with chemomechanical debridement, he believed that appropriate obturation was a critical step in maintaining healthy endodontically treated teeth. To obtain a proper seal, Schilder believed clinicians must fill the root canal space as best as they could in three-dimensional way. He demonstrated his technique through condensation of heated gutta percha increments. This method would encourage a more complete and homogenous obturation from the cementoenamel junction to the cementodentinal junction (73). Clinicians began adopting these beliefs including Ford, who advocated proper obturation allows for less space for bacterial colonization, prevents apical contamination, and prevents apical migration of bacteria along the walls of the canal. Along with proper obturation, Ford also expressed the necessity of an aseptic environment utilizing rubber dams, proper coronal restorations, and recalling endodontically treated teeth to ensure successful therapy (74). With all this advancement in understanding endodontic treatment and disease, it can be summarized that successful root canal procedures require drastic reduction of bacteria within the canal space, three-dimensional seal of the root canal space, appropriate coronal restorations to prevent coronal recontamination, and suitable recall appointments to monitor the success of the treatment.

APEXOGENESIS

Apexogenesis is a procedure that maintains vital tissue with the hope of promoting root development for immature, vital teeth. Often these teeth have been affected by caries or trauma before complete root formation (75). Apexogenesis requires the removal of the inflamed pulp tissue by performing a pulpotomy. Once hemostasis is

achieved, a pulp dressing is applied along with a permanent restoration (76). Calcium hydroxide has typically been the medicament used for apexogenesis procedures due to its biocompatibility, disinfection capability, and ability to induce tissue formation. Unfortunately, calcium hydroxide has shortcomings, which include pulpal irritation and disorganized dentin bridge formation. With the rise of bioceramics, MTA has been studied in detail and has demonstrated more consistent outcomes for apexogenesis procedures when compared to calcium hydroxide. Unlike calcium hydroxide, MTA does not stimulate pulpal irritation and does lead to complete reparative dentin bridge formation (77). The drawbacks of MTA include coronal tooth discoloration, long setting time, and a costlier product for the practitioner (78). This new wave of bioceramics has incited growth leading to several new alternatives for MTA.

After the clinician has achieved hemostasis in an aseptic environment, the bioceramic material will be placed directly on the vital tissue and sealed with an appropriate restoration. For apexogenesis, scheduling adequate recall was imperative. For immature teeth presenting with vital pulps requiring root canal treatment, apexogenesis is the recommended procedure. However, when needing to treat immature teeth with necrotic pulp tissue, the clinician should be performing apexification or regenerative endodontic procedures.

APEXIFICATION

Immature teeth with necrotic pulps have been treated with apexification procedures since the 1960s. With an open apex, conventional root canal treatment is avoided in these teeth due to various complications during treatment, especially obturation. The primary goal of an apexification procedure is to create a calcified barrier

at the apex of the tooth. To form this calcific stop, long term calcium hydroxide placement is utilized. This allows the practitioner to have a membrane to condense obturation materials against without being concerned about extrusion (11).

As with all endodontic procedures, rubber dam isolation is a requirement for apexification to ensure a dry and aseptic environment while protecting the patient. Due to the large open canal with thin dentin walls, chemical irrigation is the primary route of disinfection with minimal mechanical instrumentation. The endodontist should continue to place calcium hydroxide every three months until calcific matrix can be seen radiographically. When the calcified stop is visibly formed, it can be obturated with various materials such as gutta percha, MTA, or other bioceramic alternatives. The duration of treatment can last anywhere from nine to 24 months (75).

There are several shortcomings to this protocol that are important to consider when treatment planning. These apexification-produced barriers show a cementum-like substance with minute portals of communication with the periapical tissues. Another drawback to this procedure is it does not induce further root development in length or thickness (11, 79). Furthermore, patient compliance is a key factor in success of this treatment as therapy could last up to two years. Lastly, studies have demonstrated that long term calcium hydroxide applications can lead to weakened dentin which in turn decreases root fracture resistance (80-83).

An additional technique that requires less time commitment is creating an artificial apical plug. In this procedure, the clinician will still utilize calcium hydroxide until the patient becomes asymptomatic. Following this, an apical plug of 4 mm to 5mm is placed after the canal is cleaned and dried (84). If patient compliance is an issue, this

alternative should be considered. Since there is a shorter duration of calcium hydroxide treatment, the tooth will have a less significant reduction in fracture resistance. This technique has been shown to have success from 85 percent to 93.5 percent (85, 86). However, this procedure does not allow for continued root maturation and thus leaves the tooth with thin dentin walls more prone to fracture.

HISTORY OF REGENERATIVE PROCEDURES

Regenerative procedures can be traced back to 1961 when Nygaard-Østby attempted to promote healing through inducing a blood clot within the root canal system. This procedure was performed on 17 patients with vital or necrotic pulp tissue and included foraminal enlargement, intracanal medicament placed for the necrotic pulps, induction of intracanal bleeding, and obturation coronal to the blood clot. All teeth within a period of 17 days to 3.5 years were extracted and assessed histologically. After examination, it was found that all teeth exhibited resolution of inflammation, elimination of pathosis for the necrotic pulps, and evidence of radiographic apical closure in select teeth (87). The in-growth of connective tissue observed in the apical region was not identical to pulp and lacked certain cells including odontoblasts (88). This experiment laid the groundwork for the future of regenerative endodontics.

Following the work of Nygaard-Østby, the application of antibiotics in the canal system was used to disinfect the root canal system. Practitioners utilized a polyantibiotic dressing after instrumenting short of apical tissue in vital immature teeth. After obturation, all cases were followed and showed the resolution of symptoms with continued root development (89).

As of late, several reports are being published demonstrating continued root

development in immature teeth. The case reports often involve the use of a combination of antibiotic pastes to create an aseptic environment within the root canal system. The first of many modern regenerative endodontic procedures recorded was one by Iwaya, who used a double antibiotic paste made up of metronidazole and ciprofloxacin to disinfect an immature tooth with a necrotic pulp (90). Iwaya utilized 5-percent sodium hypochlorite and 3-percent hydrogen peroxide for chemical disinfection during the appointment and DAP as the intracanal medicament between appointments. After 6 visits and 30 months, continued root development with a positive response to sensibility testing was accomplished. Professionals began experimenting and utilizing antibiotic pastes, including Banchs and Trope, who used a triple antibiotic paste (TAP), composed of ciprofloxacin, minocycline, and metronidazole, to disinfect the root canal system. These two used sodium hypochlorite with TAP as an intracanal dressing for 28 days but did not utilize mechanical instrumentation (91). After removal of the TAP, they induced intracanal bleeding and restored the tooth coronal to the established blood clot. In this case report, Banchs and Trope were able to have resolution of periapical inflammation, continued root development, and positive response to sensibility tests. These case reports laid the foundation for the regenerative endodontic protocols utilized today.

The goals of regenerative endodontic procedures include resolution of symptoms, healing of the apical pathosis, completion of root development, and the establishment of pulpal vitality and function (92). If pulp tissue were to be completely regenerated, it would possess vascularity, innervation, analogous architecture and density to healthy pulp tissue, and the ability to create odontoblasts (93). For this to occur, the root canal system must be disinfected to create an environment capable of tissue engineering (94).

Once disinfection is achieved, regeneration requires stem cells, a scaffold, and pertinent growth factors (95).

DISINFECTION

The cause of apical periodontitis is from bacterial infection of the root canal system of a tooth with a necrotic pulp. Bacteria found within the root canal system include anaerobic gram-negative and gram-positive bacteria. The primary gram-negative bacteria species found include Fusobacterium, Dialister, Porphyromonas, Prevotella, Tannerella, Treponema, Campylobacter and Veillonella. The dominating gram-positive bacteria often found are Parvimonas, Fillifactor, Pseudoramibacter, Olsenella, Actinomyces, Peptostreptococcus, Streptococcus, Propionibacterium and Eubacterium (96). When evaluating primary endodontic infections of immature permanent teeth, Nagata et al discovered that *Actinomyces naeslundii* was the principal bacterial species present (97). The microbes exist within the canal system and attach to tooth structure by means of a biofilm, a dynamic structure made up of several bacterial species and their byproducts (98).

For most oral infections, the makeup of the bacterial biofilm varies depending on the location of the biofilm and the stage of infection. The microbial composition is influenced by factors including host defense mechanisms, nutrient availability, and oxygen saturation (99). As the oxygen supply diminishes with limited blood flow, the predominant bacterial profile shifts from facultative anaerobic bacteria to more obligate anaerobes.

The main focus of treatment for an immature necrotic tooth has always been is accomplished by means of chemical disinfection with varying irrigants and intracanal

medicaments to diminish the microbial insult. The endodontic community has favored utilizing triple antibiotic paste (TAP) for treatment of immature teeth with necrotic pulps. Studies have demonstrated that TAP is effective against bacterial species seen in infected root canals *in vitro* and *in vivo* (3, 25).

As with most medicaments, they have select drawbacks. The minocycline within TAP leaves the dentin darkly stained, which is of concern as these teeth often are present in the esthetic zone (31). Due to this challenge, professionals began experimenting with other antibiotics such as cefaclor, amoxicillin, and clindamycin (21, 100). Several studies demonstrated the effectiveness against several endodontic pathogens; clindamycin was included in a modified triple antibiotic paste (MTAP) as a substitute for minocycline (101, 102). MTAP was found to be an effective disinfectant in immature teeth with a necrotic pulp for regenerative endodontic purposes (100). Another alternative to avoid dentin staining is simply just removing the minocycline from the antibiotic paste thus forming a double antibiotic paste.

Often clinicians form a paste-like mixture of these antibiotic powders in saline for ease of application into the canal. This paste typically results in an antibiotic concentration of around 1 g/mL (32). Studies have demonstrated that this high concentration is irreversibly damaging to human stem cells of the apical papilla and dental pulp cells (32, 103, 104). Due to this harmful effect, other concentrations have been studied ranging from 0.1 mg/mL to 2 mg/mL (32, 103, 104). Outside of the cytotoxic effects of high concentration antibiotic pastes, they were demonstrated to have a negative effect on chemical and mechanical properties of radicular dentin (28, 105).

Along with interappointment intracanal medicaments, disinfection of the canal

system is completed with the aid of various irrigation solutions. Chemical disinfection of the root canal system has been shown to provide maximum benefit against bacterial species found within the canal system (106). Sodium hypochlorite has demonstrated to quickly eradicate gram-negative anaerobic rods often seen in apical periodontitis (107). Although irrigation solutions have a powerful effect on bacterial species, Estrela et al. found that sodium hypochlorite and chlorhexidine are not effective in fully removing *E. faecalis* from the root canal system. Despite this limitation, sodium hypochlorite is a primary irrigant for its ability to dissolve organic tissue and eradicate numerous bacterial species.

The efficacy of sodium hypochlorite is influenced by various properties including concentration and temperature. When the temperature and concentration is increased, sodium hypochlorite has a greater antimicrobial and tissue dissolution effect (108, 109). In fact for each temperature increase of 5°C, the bactericidal rate of sodium hypochlorite is doubled (110). Studies have demonstrated that it is safe and effective to utilize 6-percent sodium hypochlorite solutions during chemical disinfection of the root canal system (111). However, when considering chemical disinfection for regenerative endodontic procedures, the clinician must acknowledge the more open apex when compared to a fully matured tooth. With a blunderbuss apex, there is an increased risk of extrusion of the irrigation solutions. Outside of procedural accidents resulting from extrusion of the solution, high concentrations of sodium hypochlorite have been found to have a significant cytotoxic effect on stem cells (19). This is of concern when dealing with regenerative endodontic procedures because a goal is to preserve the stem cells found at the apical papilla. Furthermore, it has been discovered that sodium hypochlorite

diminishes radicular dentin's potential for the release of growth factors necessary for the differentiation of stem cells of the apical papilla (112). Due to the negative effects on stem cells with higher concentrations of sodium hypochlorite, the American Association of Endodontics suggests using a reduced concentration of 1.5-percent sodium hypochlorite for regenerative endodontic procedures (113).

Another favorite irrigant solution clinicians often use is ethylenediaminetetraacetic acid (EDTA), which is a chelating agent that removes the inorganic matter of a smear layer (114). A smear layer is formed through the packing of dentin shavings into dentinal tubules during mechanical preparation of a root canal. When dentinal tubules are burnished and occluded with dentin shavings, the sealing ability of root canal sealers is diminished. However, it has been demonstrated that EDTA can remove this smear layer, which leads to a greater seal of the root canal system (115). Studies have shown that a one-minute rinse with EDTA within the canal system will effectively remove the smear layer (75).

The American Association of Endodontics includes EDTA in their suggested protocol for regenerative endodontic treatment for differing reasons than smear layer removal, as there is minimal mechanical preparation. It was demonstrated that 17-percent EDTA possesses less cytotoxic effects when compared to 6-percent sodium hypochlorite and 2-percent chlorhexidine. Two-percent chlorhexidine was shown to be the most cytotoxic to viable cells, which is why several protocols do not include its use in their procedures (116). EDTA has further exhibited the ability to increase adherence of newly produced mineralized tissue to dentin walls (117). Furthermore, EDTA releases growth factors embedded in the dentinal wall during dentinogenesis, which promotes the

differentiation of stem cells from the apical papilla (92).

STEM CELLS

Stem cells are classified into two primary categories depending upon their differentiation potential: pluripotent and multipotent stem cells. Pluripotent cells have the ability to differentiate into any possible cell lines, whereas multipotent cells can only differentiate into specific and limited cell lineages. Moreover, stem cells can be further classified into three groups dependent upon their source: autologous, allogeneic, and xenogeneic. Autologous stem cells are ones derived from the same individual utilizing the cells. Allogeneic stem cells are those from an organism of the same species. The xenogeneic types are cells from an entirely separate species.

From the work of Lovelace, it has been discovered that after induction of bleeding into the canal system, autologous stem cells are delivered locally (2). These autologous, multipotent stem cells have the capability to differentiate into several specific cells of the body. Dental pulp stem cells (DPSCs) (118), stem cells from human exfoliated deciduous teeth (SHEDs) (119), periodontal ligament stem cells (PDLSCs) (120), dental follicle progenitor stem cells (DFPCs) (121), and stem cells from apical papilla (SCAPs) (122, 123) have all been found to be sources for these multipotent stem cells. DPSCs are located adjacent to the odontoblastic layer, approximating the cell rich zone (75). Due to its ability to differentiate into mature odontoblasts, DPSC has been extensively studied for regenerative endodontics (124). SCAPs have also been investigated for regenerative endodontics due to the capability of dentin formation (123, 125).

SCAFFOLD

A scaffold has various purposes in regenerative endodontics including providing an environment for cell growth, stem cell differentiation, and formation of new vasculature (92). In 1976, Nevins presented the idea of using a collagen gel scaffold for regeneration procedures (126). Following this, Thibodeau found benefits of the formation of a blood clot for regeneration procedures (127). He compared the revascularization potential of a collagen scaffold, a blood clot scaffold, and the combination of the two. It was shown that both models with a blood clot had more success than a collagen scaffold alone. This finding led to the belief that a scaffold is not only critical to act as a physical matrix for regeneration but also to contain vital endogenous growth factors.

Furthermore, Hutmatcher (128) went on to describe ideal qualities of scaffold for regeneration purposes:

1. Porous structure for tissue and vascular integration.
2. Biodegradable at a rate of tissue formation.
3. Allow cellular attachment for differentiation and proliferation.
4. The mechanical properties of the site being implanted must be adequate.
5. Does not elicit any adverse reactions.
6. Easily formed into different sizes and shapes.

Most regenerative endodontic procedures use a blood clot as a scaffold from lacerating the apical papilla (92). Other scaffolds have been studied including using platelet-rich plasma and platelet-rich fibrin to provide additional growth factors (4, 116, 129).

GROWTH FACTORS

Growth factors include a wide-ranging group of endogenous molecules that encourage cell growth, healing, and maturation, which is of great benefit for regeneration of pulp like tissue in these procedures. Studies have demonstrated that various growth factors can be embedded within the dentinal wall during dentinogenesis (130). For pulp regeneration, there are numerous beneficial growth factors including bone morphogenic protein (BMP), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF). BMPs have been found to encourage odontoblast differentiation. TGF-β allows for the promotion of odontoblast differentiation, pulp tissue mineralization, wound healing, and anti-inflammatory signaling. Lastly, VEGF has been shown to regulate angiogenesis through inducing chemotaxis, proliferation, and differentiation of cells for vascular ingrowth and dental pulp cells (130).

CLINICAL DECISION-MAKING

The clinician must be cognizant of several factors when planning for the treatment of an immature tooth with a necrotic pulp such as patient desires, patient compliance, outcome data, dentin wall thickness, and many more. When evaluating apexification procedures, it was found that long-term application of calcium hydroxide exhibited success rates from 74 to 100 percent, whereas an MTA apical stop has shown success rates over 90 percent (85, 131, 132). Jeeruphan et al. compared 22 calcium hydroxide apexification cases, 19 MTA apexification cases, and 20 revascularization cases. When evaluating outcomes in this retrospective study, it was observed that 77.2 percent of calcium treated teeth were deemed successful, 95 percent of MTA apexification showed success, and 100 percent of revascularization cases were successful. Revascularization

cases without pulpal regeneration were still identified as successful if the tooth was retained at follow up. Moreover, the revascularization cases statistically exhibited a greater increase in root length and thickness compared to the MTA and calcium treated cases (12). With greater thickness of dentin walls, there is a reduced risk of fracture for the compromised teeth thus an increased survival rate. Furthermore, Jeeruphan found root fractures in 23 percent of the teeth treated with calcium hydroxide, which is in agreement with other studies showing that long-term calcium hydroxide application is associated with an increased risk of fracture (12, 26, 82)

There has been a growing interest in the field of regenerative endodontics for the dental community and it is imperative that this interest continues to prosper and is backed by stronger research. Most published data on the subject are case reports with no randomized clinical trials or meta-analyses available to further strengthen the field of regenerative endodontics. Case reports can be beneficial in initiating awareness but do not analyze collected data to provide clinicians with evidence-based treatment protocols (21). Moreover, case reports can often be biased because only successful clinical cases are ones that are published. The AAE collected and analyzed the available evidence for regeneration endodontics to provide professionals with an updated guide and protocol for treatment of these select cases. With more and more interest in this field, stronger evidence will emerge providing clinicians with the best possible data to make informed treatment decisions.

MATERIALS AND METHODS

HUMAN TEETH SELECTION

Extracted permanent molars will be collected and stored in 0.1-percent thymol solution at 4°C following approval from the Indiana University Institutional Review Board. All teeth used will be visually inspected to exclude any teeth with caries, restorations, hypocalcification, hypoplasia, cracks and dental fluorosis.

HUMAN DENTIN SPECIMEN PREPARATION

The crowns of the molar teeth will be removed at the CEJ with the root sectioned in half along the long axis using a water-cooled saw. The root halves will be prepared into dentin slabs with dimensions of 4x4x1 mm. The pulpal wall side will be polished using a Struers Rotopol 31/Rotoforce 4 (Struers, Cleveland, OH) polishing unit utilizing 500 SiC, 1200 SiC, and 2400 grit SiC abrasive papers. The dentin slab specimens will be sonicated with 1.5-percent NaOCl and 17-percent EDTA for 4 minutes to remove the smear layer as performed in an earlier study (48). To avoid dehydration, the dentin samples will be stored in water.

ANTIBIOTIC PASTE PREPARATION

The radiopaque DAP will be formulated in 3 concentrations as shown in previous studies (37, 38) with small alterations to include a radiopaque filler into the DAP. Briefly, 10 mg, 100 mg and 250 mg of DAP (Champs Pharmacy, San Antonio, TX) will be mixed with 10 mL of sterile water independently to create 1 mg/mL, 10 mg/mL and 25 mg/mL of DAP solutions, respectively. Following this, 3 g of barium sulfate will be incorporated

gradually to the DAP solutions while stirring to create a 30-percent radiopaque DAP slurry made up of 30% (w/v) of barium sulfate. Thereafter, 0.7 g of methylcellulose powder (Methocel 60 HG, Sigma-Aldrich, St. Louis, MO) will be slowly incorporated into each DAP slurry at room temperature producing a pasty consistency of DAP. Lastly, the solution will be centrifuged for 15 minutes at 7000 rpm to create a homogenous injectable paste absent of air bubbles with 1 mg/mL, 10 mg/mL, and 25 mg/mL of DAP.

HUMAN DENTIN SPECIMEN TREATMENT

For the current study, 140 dentin samples will randomly be assigned to 14 experimental groups (n = 10 per group). After being sterilized in ethylene oxide, each dentin sample will be placed in one well of a 96- sterile-well plate with the pulpal side facing up. Each specimen will be treated for 1 week or 4 weeks, which will be assigned randomly. Within these treatment times, there will exist five separate treatment groups consisting of 200 μL of the following: Group 1 – 25 mg/mL of DAP with radiopaque filler, Group 2 – 10 mg/mL of DAP with radiopaque filler, Group 3 – 1 mg/mL of DAP with radiopaque filler, Group 4 – radiopaque paste without DAP, Group 5 – $Ca(OH)_2$, Groups 6 – Sterile water with bacterial biofilm, Group 7 – Sterile water.

Following treatment, each dentin sample will be irrigated with 5 mL of sterile saline for 1 minute. Then the samples will be dried using sterile gauze before being irrigated with 17-percent EDTA for 5 minutes. Following the irrigation of 17-percent EDTA, the specimens will then be washed with 5 mL sterile water for 1 minute. All of them will be placed in individual wells with 200-μl phosphate buffered saline (PBS) at 37˚C for 1 week before analyzing the antibacterial effects.

BACTERIAL COLLECTION

A bacterial isolate will be obtained from an immature tooth with necrotic pulp that was treated or scheduled to be treated using a regenerative endodontic procedure (IRB #1510640949). During the endodontic procedure, the tooth and rubber dam will be rinsed with 3.0-percent hydrogen peroxide and disinfected with 6.0-percent sodium hypochlorite. Sterile burs will be utilized to perform traditional access of the cavity. Following coronal access, the chamber of the pulp will be disinfected with a swab saturated with 6.0-percent sodium hypochlorite then deactivated using sterile 5.0-percent sodium thiosulfate. The bacterial specimen will be collected from the necrotic canal system utilizing a #15 K-file. The file will be advanced to 1 mm short of the apical foramen and a filing action will be performed for 30 seconds. Three sterile paper points will be introduced to the same working length and remain in the canal system for 1 minute to adequately absorb tissue fluid containing bacteria. The file and paper points will be put into a previously made solution of 2 mL of BHI-YE supplemented with Vitamin K/Hemin. Then, it will be vortexed, grown anaerobically at 37˚C for 48 h and frozen at -80˚C until used.

BACTERIAL GROWTH ON TREATED ROOT SPECIMENS

After 1 week in PBS, the dentin samples in 12 of the 14 groups mentioned earlier will be transferred to a new 96-well sterile plate containing 190 µl of fresh BHI-YE supplemented with Vitamin K/Hemin growth media. Then, 10 µl of an overnight multispecies bacterial culture (10^6 colony forming units (CFU/mL)) will be added to each well and incubated anaerobically for three weeks at 37°C. Throughout the biofilm maturation phase, the media will be replaced every seven days. It is worth noting the

inability of using anaerobic chamber in the proposed study may lead to the absence of anaerobic species. Thus, only facultative, capnophilic and aerobic bacteria in the isolate will be tested. One group from each time point will not be subjected to bacterial infection and will undergo regular replacement of fresh media (sterile control). The biofilm will be analyzed utilizing scanning electron microscopy one day and three weeks after dentin exposure to confirm the polymicrobial nature of the formed biofilm. After a three-week incubation to permit biofilm formation (49), sterile saline will be used to rinse each sample twice to eliminate unattached bacteria. The samples will be moved to individual sterile plastic tubes containing 200 µl of sterile saline and sonicated and vortexed for 30 seconds each to remove biofilm bacteria. These detached bacterial biofilm cells will be diluted and spirally plated on blood agar plates (CDC, BioMerieux). Following spiral plating, incubation will be completed in 5.0-percent CO_2 at 37°C for 48 hours. The blood agar plates will be removed and colonies will be counted (CFUs/mL) utilizing an automated colony counter (Synbiosis, Inc., Frederick, MD).

STATISTICAL ANALYSIS

The effects of treatment and time on bacteria counts will be analyzed using a two-way ANOVA followed by pair-wise comparisons using Fisher's Protected Least Significant Differences to control the overall significance level at 5 percent. The bacteria counts are expected to follow a log-normal distribution, so the analyses will be performed using the logarithm of the counts. If the data are not log-normal, nonparametric tests will be used

.

SAMPLE SIZE

Based on previous data, the coefficient of variation is estimated to be 0.6. With a sample size of 10 per group, the study will have 80-percent power to detect a 2.1x difference in means between any two groups, assuming two-sided tests each conducted at a 5-percent significance level.

CLINICAL SIGNIFICANCE

With the help of several previous studies at IUSD, an optimal concentration of DAP has been shown to have ideal antibacterial effects. When adding a radiopaque filler, it is necessary to confirm that the antibacterial effects of DAP have been left unchanged. Following this study, radiopaque DAP will be closer to being a commercially available product that could aid in regenerative endodontic procedures across the world.

RESULTS

BIOFILM VALIDATION

A scanning electron microscope was utilized to confirm the polymicrobial nature of the biofilm. The biofilm used in this study was from an immature tooth with pulpal necrosis and was grown anaerobically for three weeks. As shown in Figure 21, the biofilm demonstrates multiple, varied species present on the dentin surface including rod and cocci-shaped bacteria.

RESIDUAL ANTIBACTERIAL EFFECTS OF TREATMENTS

The mean of the $\log_{10}$ CFU/mL values of the tested groups for the 1-week treatment group are as follows: 0.00 for 25-mg DAP, 1.00 for 10-mg DAP, 4.84 for 1-mg DAP, 6.35 for calcium hydroxide, 6.34 for placebo, 6.30 for no treatment. The mean of the $\log_{10}$ CFU/mL values of the tested groups for the 4-week treatment group are as follows: 0.00 for 25-mg DAP, 0.69 for 10-mg DAP, 4.45 for 1mg DAP, 6.17 for calcium hydroxide, 5.98 for placebo, 6.09 for no treatment. These data results are summarized in Table I and displayed in Figure 22.

COMPARING THE EFFECTS OF MATERIALS AND TREATMENT TIMES

Materials played a significant role on the residual antibacterial effect of the treated dentin samples. Calcium hydroxide had significantly higher bacterial counts than 1-mg DAP, 10-mg DAP, and 25-mg DAP ($p = 0.001$). When compared to the placebo and no treatment group, calcium hydroxide was not significantly different. With 25-mg

DAP, there was a significantly lower bacterial count when compared to all other treatment groups ($p = .001$). When investigating 10-mg DAP, there was a significantly lower bacterial count for all other materials except 25-mg DAP ($p = 0.001$). There was a significantly lower bacterial count for 1-mg DAP when compared to all treatment groups besides 10-mg DAP and 25-mg DAP in which there was a significantly higher bacterial count ($p = 0.001$). The placebo group and no treatment group did not have a significant difference in bacterial count. Treatment time had a significant effect on the residual antibacterial effect. The bacterial counts at 4 weeks were significantly lower that the counts at 1 week ($p = 0.0105$). This data can be found summarized in Table II.

FIGURES AND TABLES

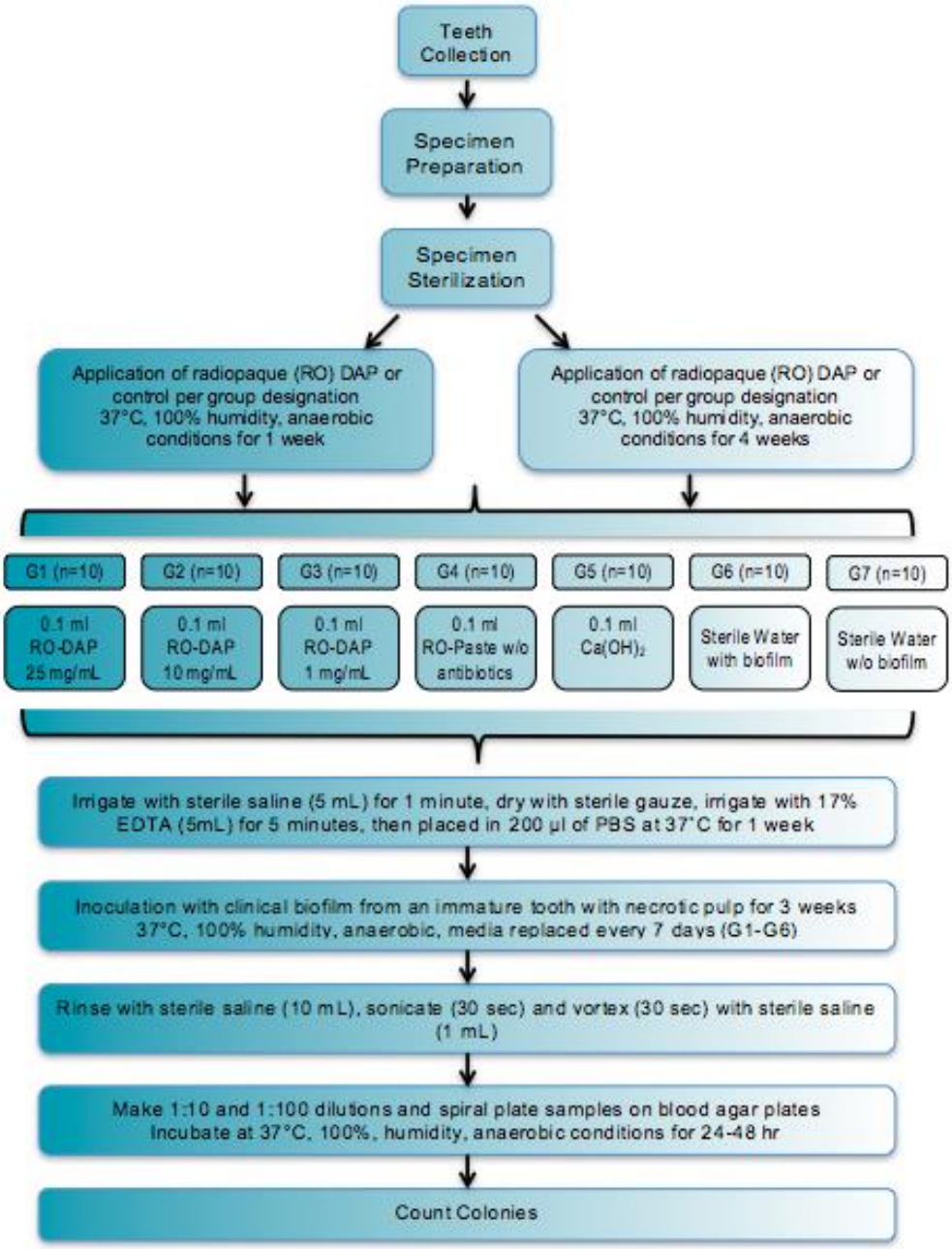

FIGURE 1. Flowchart of experimental methodology.

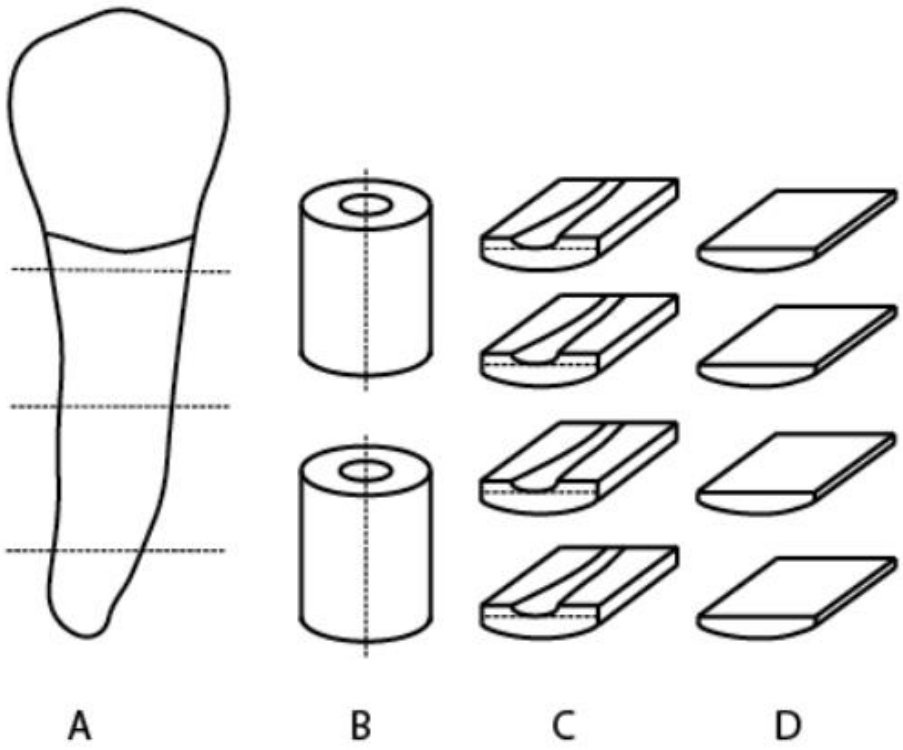

FIGURE 2. Roots (A) were sectioned, cut longitudinally (C), and polished flat on the pulpal side (D).

FIGURE 3. Saw used to initially section whole teeth.

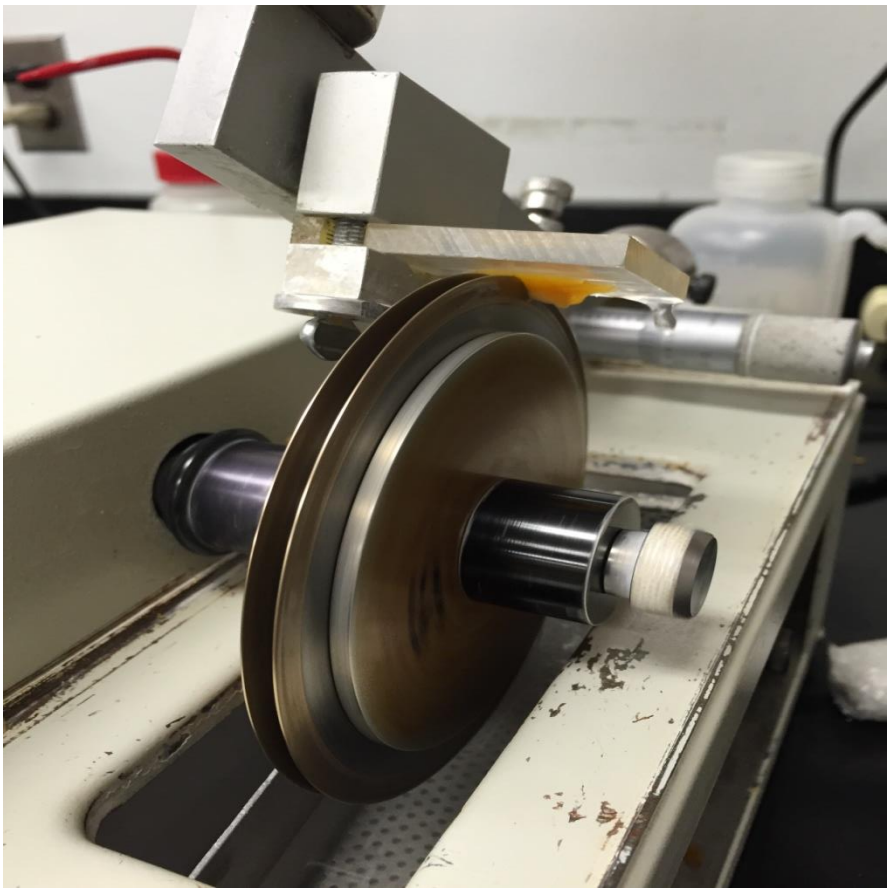

FIGURE 4. Low-speed saw used to section teeth into 4x4x1-mm samples.

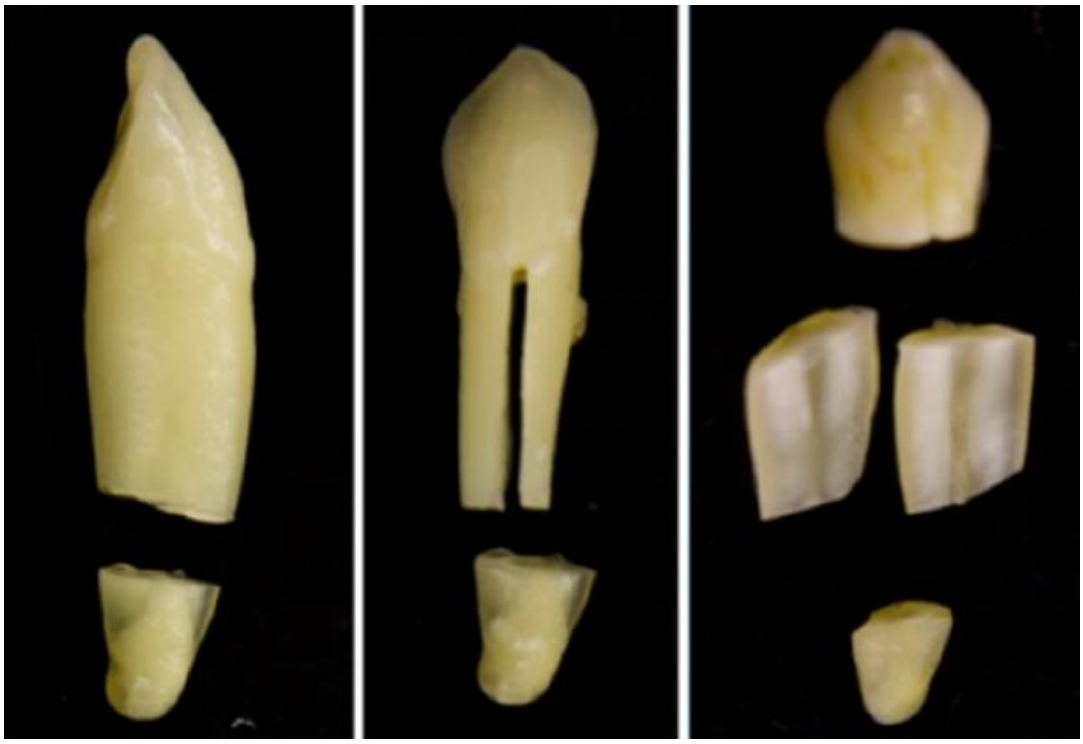

FIGURE 5. Example of properly sectioned tooth.

FIGURE 6. 4x4-mm dentin samples mounted on polishing jig.

FIGURE 7. Dentin polishing unit.

FIGURE 8. Polishing discs.

FIGURE 9. Example of polished 4x4-mm dentin sample.

FIGURE 10. Example of sterilized 4x4-mm dentin sample.

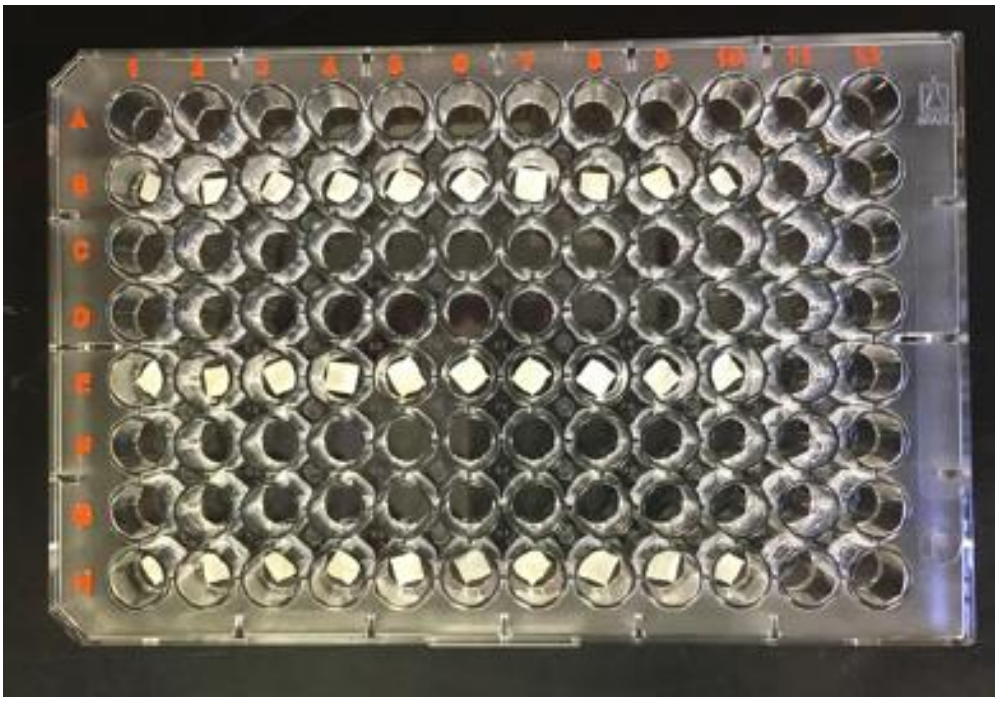

FIGURE 11. Dentin specimens placed into sterile 96-well plate.

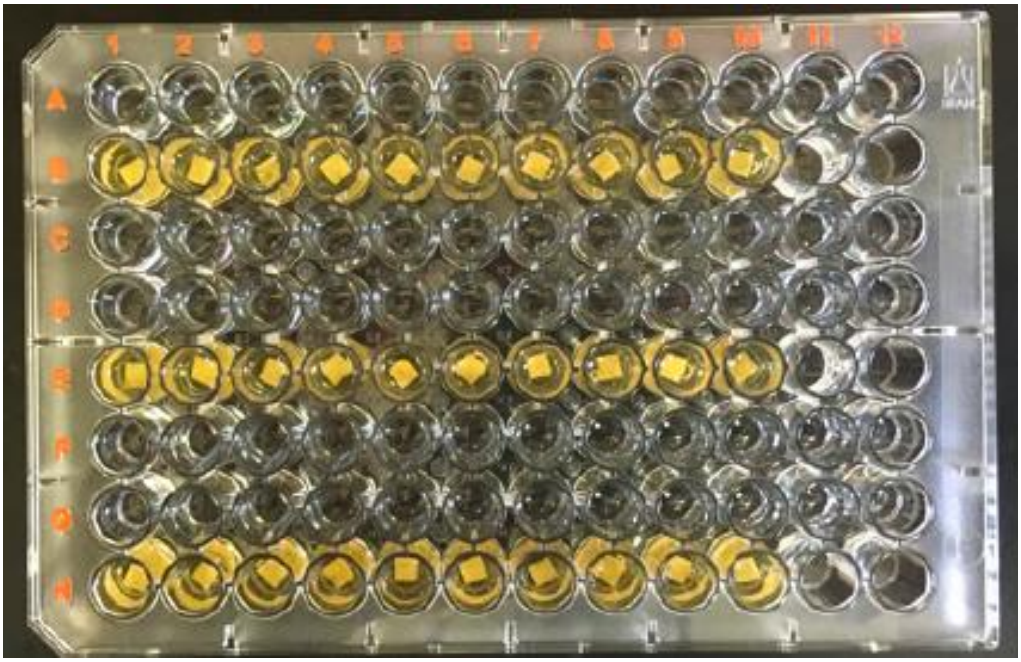

FIGURE 12. Uninoculated dentin specimens with BHI-YE broth supplemented with Vitamin K/Hemin.

FIGURE 13. All dentin specimens were pretreated for 1 week and 4 weeks at 37°C with 100% humidity in the above incubator prior to bacterial inoculation.

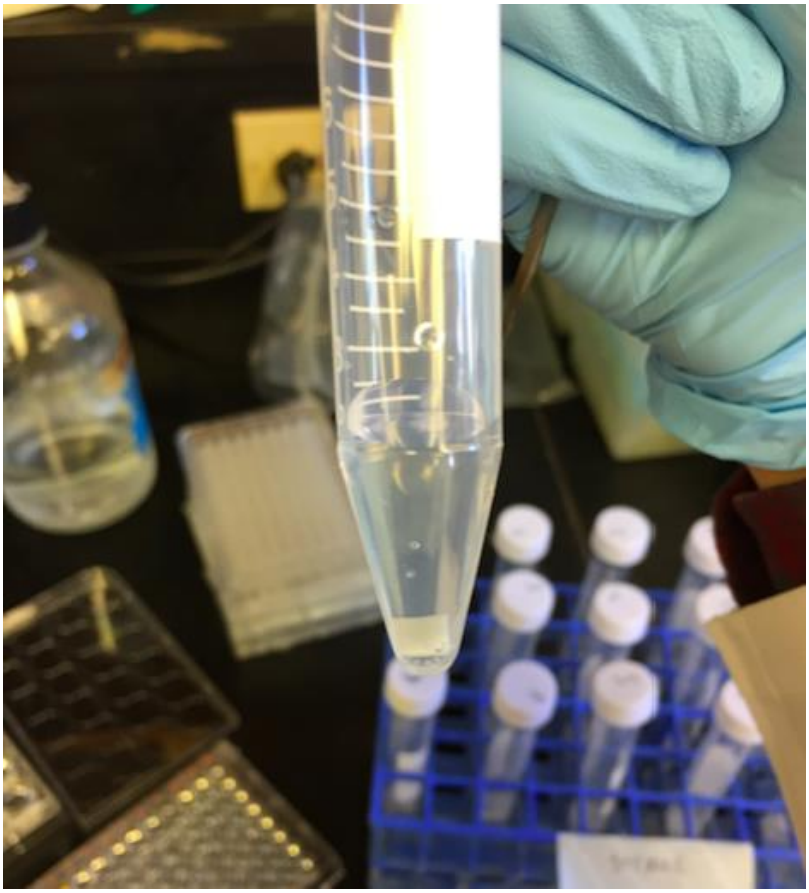

FIGURE 14. Dentin samples were placed in sterile tubes after 3-week biofilm grown on the specimens was rinsed.

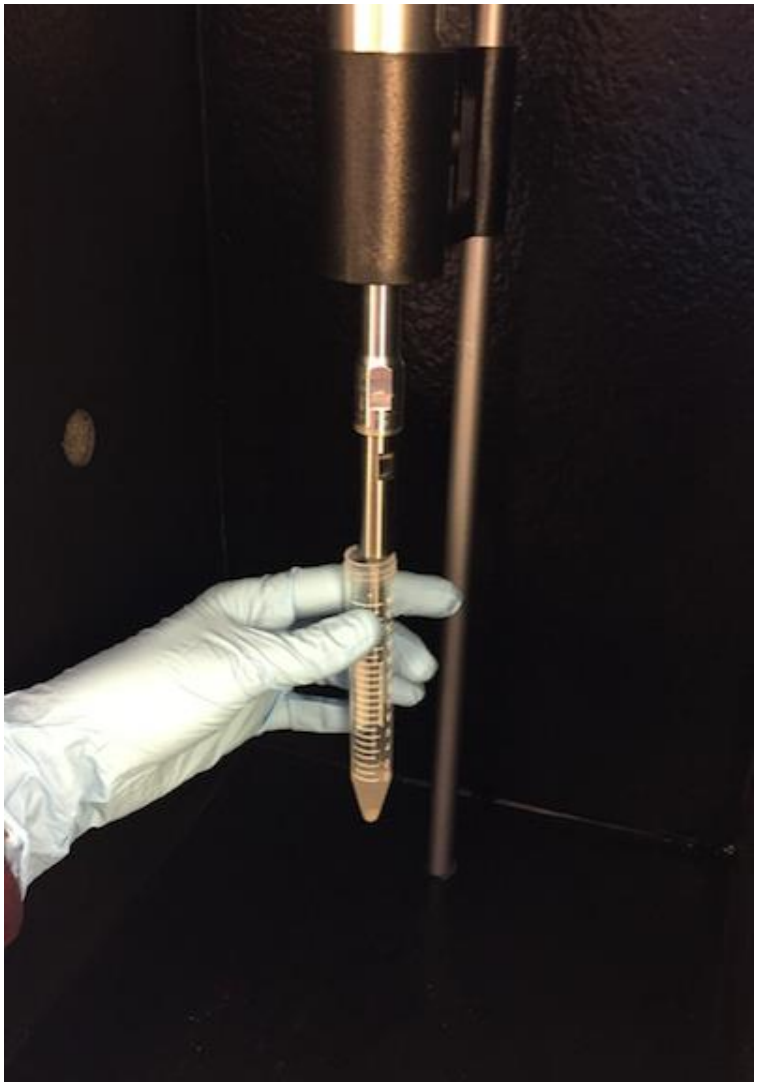

FIGURE 15. All dentin specimens were sonicated to detach biofilm cells.

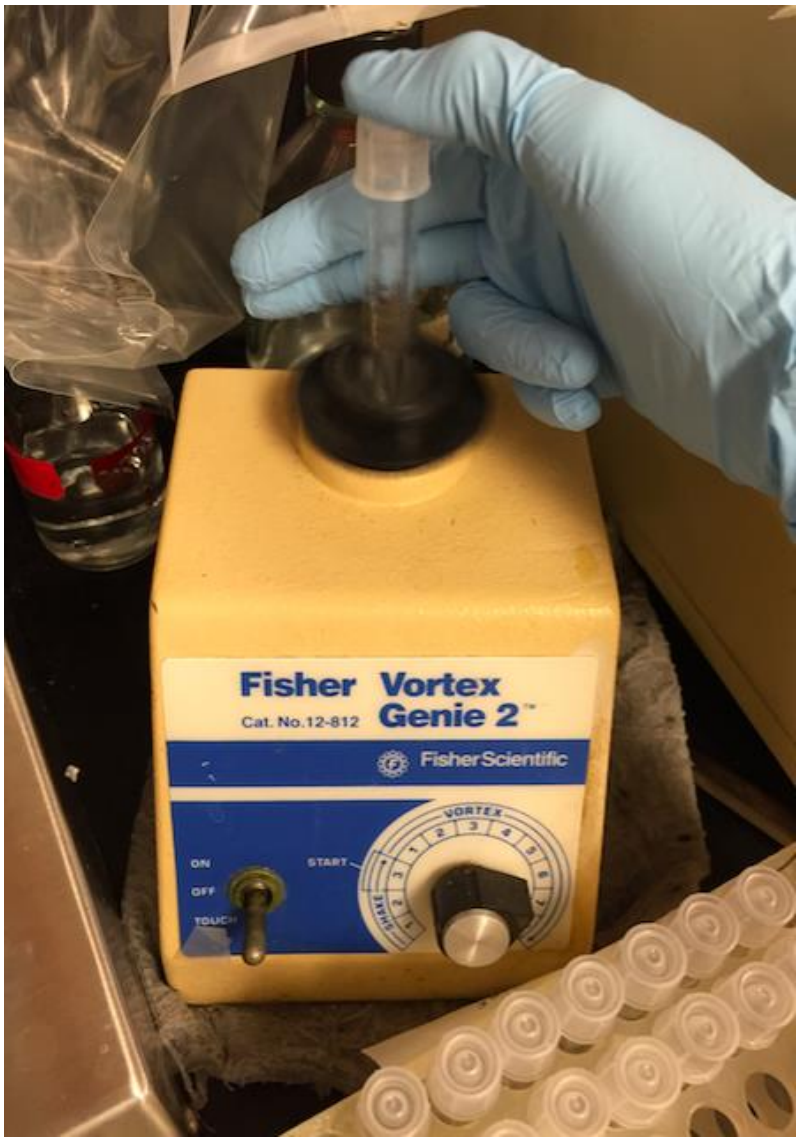

FIGURE 16. All dentin specimens were vortexed to further detach biofilm cells.

FIGURE 17. Spiral plating after dilutions of detached biofilm cells.

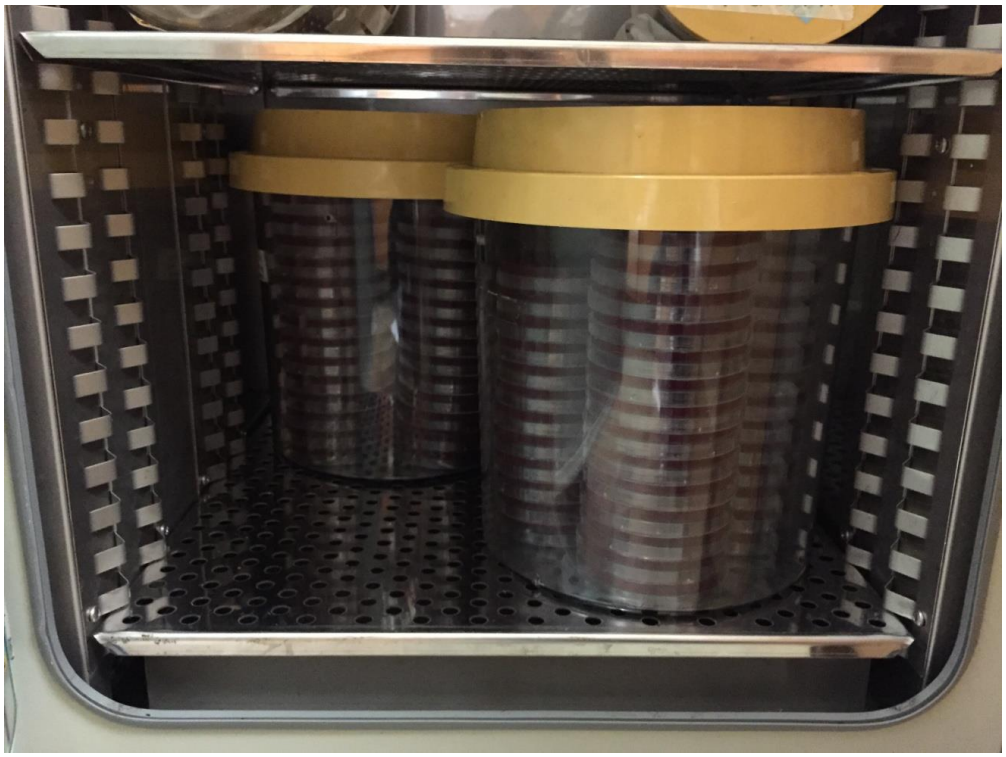

FIGURE 18. Spiral plates were incubated anaerobically for 48 hours.

FIGURE 19. Blood agar plates following 48 hours of incubation after spiral plating.

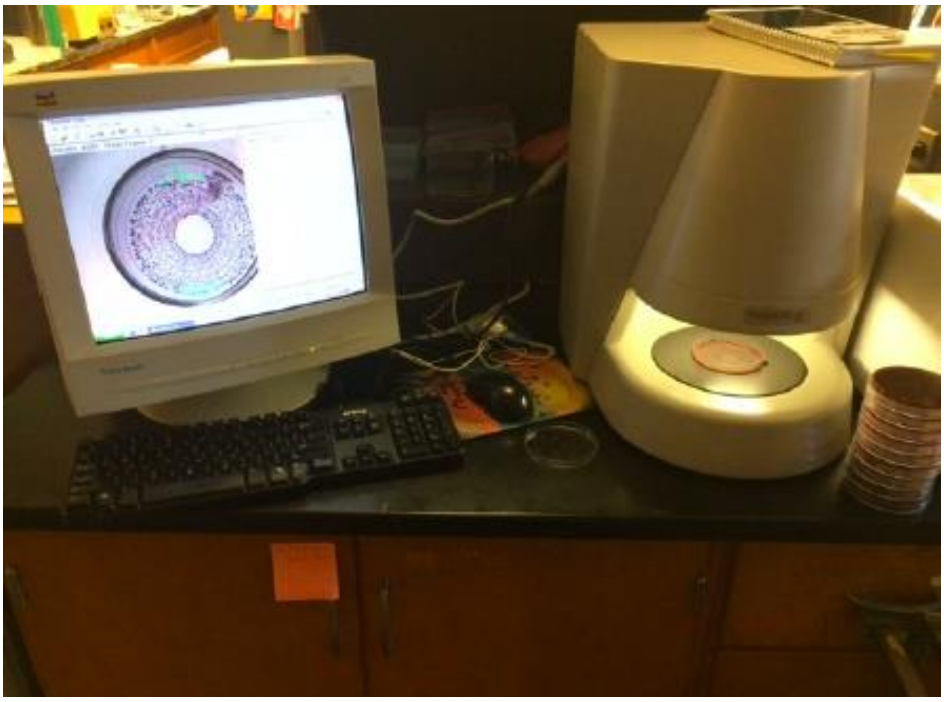

FIGURE 20. Blood agar plates were placed in an automated colony counter after 48-hour incubation period to enumeration CFU/mL.

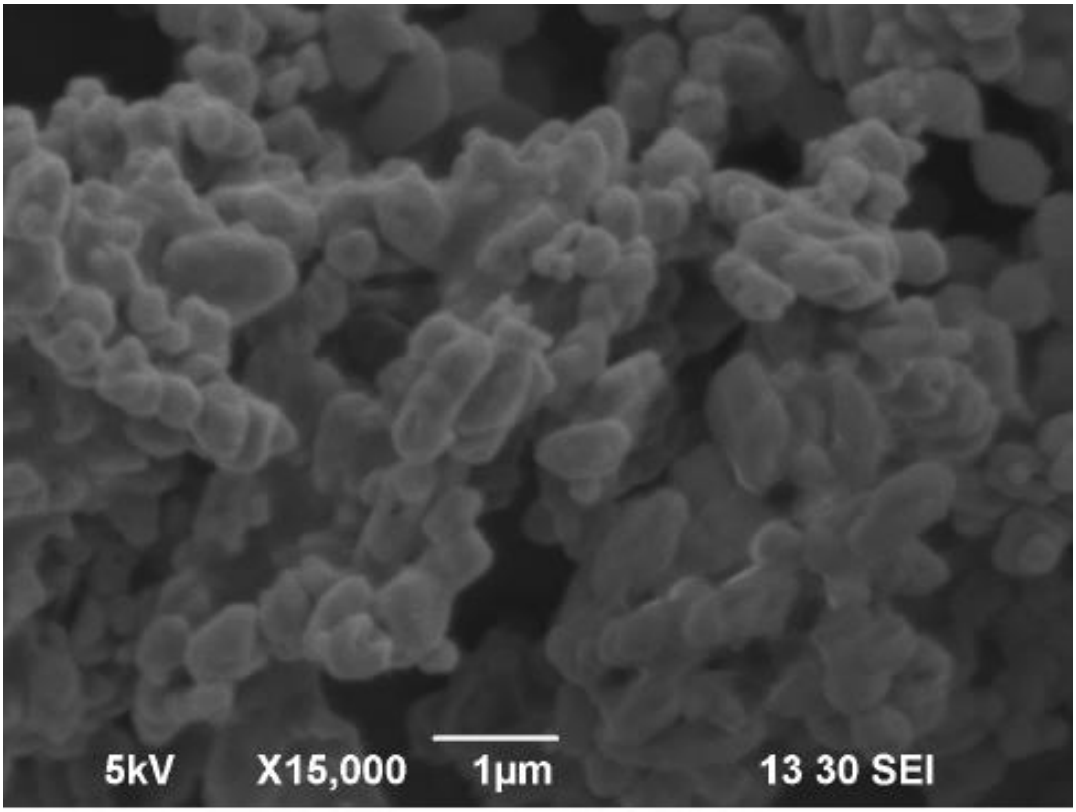

FIGURE 21. Scanning electron microscopic image of 3-week old bacterial biofilm formed on dentin surface. Biofilm formed by bacteria obtained from infected root canal of immature tooth with diagnosis of pulpal necrosis.

TABLE I

Summary showing the mean log values for each medicament at each time point

Analysis Variable: Counts										
Time	**Material**	**N**	**Mean**	**Std Dev**	**Std Error**	**Min**	**Lower Quartile**	**Median**	**Upper Quartile**	**Max**
One week	DAP 25 mg/mL	10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	DAP 10 mg/mL	10	1.0010	1.2995	0.4109	0.0000	0.0000	0.0000	2.3080	2.7852
	DAP 1 mg/mL	10	4.8418	0.1218	0.0385	4.6320	4.7568	4.8738	4.9509	4.9641
	Calcium hydroxide	10	6.3501	0.0787	0.0249	6.2429	6.2878	6.3426	6.4215	6.4610
	Placebo	10	6.3440	0.0540	0.0171	6.2679	6.2919	6.3407	6.3831	6.4302
	No treatment	10	6.2985	0.0553	0.0175	6.2097	6.2624	6.2975	6.3415	6.3837
Four week	DAP 25 mg/mL	10	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	DAP 10 mg/mL	10	0.6926	1.1152	0.3527	0.0000	0.0000	0.0000	2.3080	2.3091
	DAP 1 mg/mL	10	4.4453	0.2950	0.0933	4.0032	4.1685	4.4734	4.6738	4.8593
	Calcium hydroxide	10	6.1749	0.1796	0.0568	5.9127	6.0013	6.2508	6.3244	6.3773
	Placebo	10	5.9759	0.0917	0.0290	5.8531	5.9136	5.9671	6.0153	6.1402
	No treatment	10	6.0938	0.1504	0.0476	5.8971	5.9906	6.0692	6.2256	6.3232

TABLE II

Tabulated comparison between all medicaments as well as the time points with significance

Comparison	Result	Estimate	Std Err	Probt	Sig
Time	Four week < One week	-0.2422	0.09294	0.0105	*
Material	Calcium hydroxide > DAP 10 mg/mL	5.4157	0.1610	<.0001	*
Material	Calcium hydroxide > DAP 1 mg/mL	1.6189	0.1610	<.0001	*
Material	Calcium hydroxide > DAP 25 mg/mL	6.2625	0.1610	<.0001	*
Material	Calcium hydroxide & No treatment n.s.	0.06635	0.1610	0.6810	
Material	Calcium hydroxide & Placebo n.s.	0.1026	0.1610	0.5253	
Material	DAP 10 mg/mL < DAP 1 mg/mL	-3.7968	0.1610	<.0001	*
Material	DAP 10 mg/mL > DAP 25 mg/mL	0.8468	0.1610	<.0001	*
Material	DAP 10 mg/mL < No treatment	-5.3493	0.1610	<.0001	*
Material	DAP 10 mg/mL < Placebo	-5.3131	0.1610	<.0001	*
Material	DAP 1 mg/mL > DAP 25 mg/mL	4.6436	0.1610	<.0001	*
Material	DAP 1 mg/mL < No treatment	-1.5526	0.1610	<.0001	*
Material	DAP 1 mg/mL < Placebo	-1.5163	0.1610	<.0001	*
Material	DAP 25 mg/mL < No treatment	-6.1961	0.1610	<.0001	*
Material	DAP 25 mg/mL < Placebo	-6.1599	0.1610	<.0001	*
Material	No treatment & Placebo n.s.	0.03623	0.1610	0.8224	

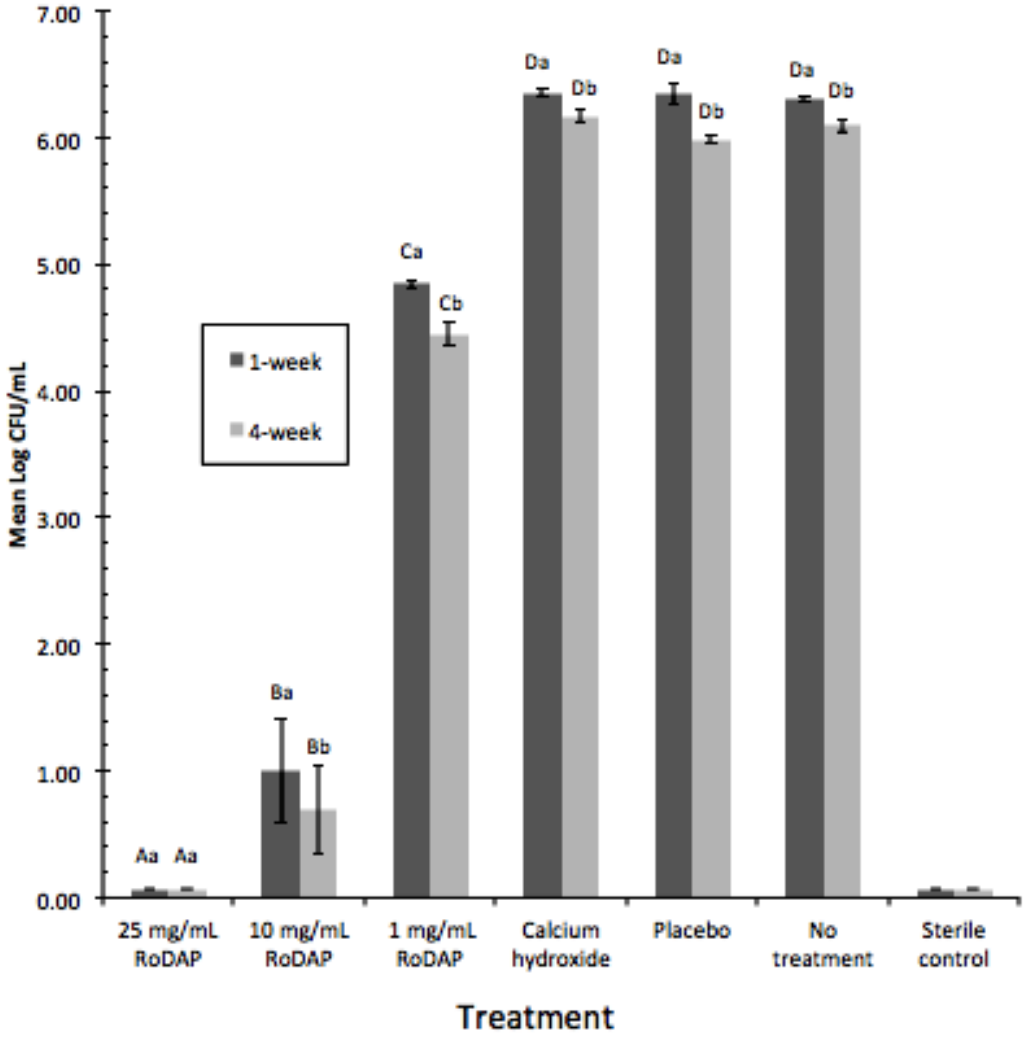

FIGURE 22. Bar graph representing results for residual antimicrobial effect of RoDAP on a polymicrobial biofilm isolated from an immature tooth with necrotic pulp. *Different capital letter denotes significant difference between medicament **Different lower case letter denotes significant difference between time

DISCUSSION

For decades, dental professionals have struggled with the treatment of an immature tooth with a necrotic pulp. Apexification procedures are often utilized to maintain these teeth but will still leave the tooth prone to complications such as fracturing. However, with the rise of regenerative endodontics, professionals have continually demonstrated the ability to increase dentin wall thickness and length thus eliminating the concern of compromised, thin dentinal walls prone to fracture. For regenerative procedures, chemical disinfection is a paramount element to ensure success. It is critical to adequately reduce the microbial load within the canal through chemical disinfection while not damaging vital components necessary for regeneration purposes.

With the growing interest in regenerative endodontics, several studies have been published focusing on the appropriate intracanal medicament and concentration. A delicate balance exists between increasing the concentration of an antibiotic medicament to a level that eliminates bacteria while avoiding detrimental effects on the apical papilla and many key components. Numerous *in-vitro* studies are utilizing reduced concentrations of various antibiotic combinations that demonstrate adequate disinfection without having a cytotoxic effect on vital stem cells and growth factors (37, 39, 133). Currently, the AAE suggests using calcium hydroxide or 0.1 mg/mL to 1.0 mg/mL of TAP or DAP as the intracanal medicament for regenerative endodontic procedures (134). This study investigated the residual antimicrobial effect of various concentrations of radiopaque DAP against a biofilm clinically isolated from an immature tooth with pulpal necrosis.

In several facets of dentistry, it is of great value to visualize the completeness of a procedure via radiographs whether it be evaluating a clinical crown margin or ensuring a densely obturated root canal system. In endodontics, radiopaque fillers are often added to intracanal medicaments to confirm via radiographs the adequate placement of a material within a canal. However, in regenerative endodontics, there are no commercially available products utilizing a radiopaque filler to verify the placement of an antibiotic paste on a radiograph. Barium sulfate is FDA-approved and often is incorporated into different medicaments to provide radiopaque properties within the materials. It can be found in many commercially available products routinely used in endodontics such as UltraCal (Ultradent, South Jordan, UT). Barium sulfate, being an inexpensive radiopaque filler, is widely used for numerous reasons. Several studies have been published focusing on the radiopaque nature of barium sulfate and demonstrate that it possesses a higher radiopacity than dentin (135, 136). Furthermore, barium sulfate has been shown to be biocompatible and non-toxic to various tissues including human dental pulp cells and mouse fibroblast cells (137, 138). Due to these reasons, barium sulfate was selected to be incorporated into the DAP investigated in this study.

For successful pulpal regeneration to occur, it is imperative to disinfect the root canal system to allow for repopulation by stem cells from the apical papilla. It has been discovered in immature teeth from young patients, bacteria seem to advance deeper into dentinal tubules when compared with adult teeth (139). Moreover, as shown by Jacobs et al., biofilms from immature teeth pretreated with DAP exhibited greater resistance to disinfection when compared with biofilms from mature teeth (140). Another study demonstrated that regardless of the concentration of NaOCl used, immature teeth exhibit

a greater resistance to disinfection than mature teeth (141). It could be assumed that this greater resistance may be due to different endodontic pathogens present in immature teeth with necrotic pulps. Nagata presented the most commonly isolated bacterial species within immature teeth with necrotic pulps are *A. naeslundii* and *Porphyromonas endodontalis* (142), whereas mature teeth with necrotic pulps show Fusobacterium and Prevotella species to be the most frequently found bacterial species (143). Futhermore, a study by Fouad indicated the presence of residual bacteria remaining in the canal system, could challenge the radiographic success of regeneration procedures (144). Additionally, Fouad suggested that small concentrations of residual antibiotics could have a beneficial effect in the success of these cases (145). Jacobs et al found that 5 mg/mL DAP provided substantial and significant residual antibacterial effects against bacterial biofilms isolated from an immature tooth with a necrotic pulp (140). Due to the clear importance of achieving chemical disinfection within the root canal system of immature teeth, this study focused on investigating the residual antimicrobial effect of a radiopaque DAP on bacterial isolates from an immature tooth with a necrotic pulp.

This study examined the residual antimicrobial effects of radiopaque DAP in concentrations of 1 mg/mL, 10 mg/mL, and 25 mg/mL. As previously mentioned, it is critical to utilize a concentration great enough to eliminate endodontic pathogens while maintaining cell vitality of stem cells found in the apical papilla. All tested concentrations exhibited a significant reduction of bacterial counts for substantivity. Since the AAE guidelines recommend using 1 mg/mL of antibiotic paste for regeneration procedures (134), 1 mg/mL of radiopaque DAP was selected for this study to evaluate the residual antimicrobial efficacy against bacterial isolates from an immature tooth with a necrotic

pulp. This study demonstrated that dentin samples pretreated with 1 mg/mL of radiopaque DAP showed significantly lower bacterial counts when compared with $(OH)_2$, radiopaque paste without antibiotics, and a no-treatment group. A study by Jenks et al. revealed that concentrations of 50-mg/mL DAP and 500-mg/mL DAP provided a significant and substantial residual antimicrobial effect against *E. faecalis* (146), while Jacobs et al. demonstrated that 5-mg/mL DAP was effective in significantly providing a residual antimicrobial effect on a biofilm clinically isolated from an immature tooth with a necrotic pulp (140). However, these studies did not incorporate a radiopaque filler into DAP; thus, this study was undertaken to confirm the residual antimicrobial efficacy of DAP with the integration of barium sulfate.

This study demonstrated that $Ca(OH)_2$ was ineffective in providing a residual antibacterial effect against clinically isolated biofilms, which has been shown by previous works by Jacobs et al. and Jenks et al. (140, 146). Numerous publications have confirmed that the antibacterial capacity of $Ca(OH)_2$ is dependent upon pH (23). This antimicrobial property can be attributed to the extreme alkaline environment created having a pH greater than 11 that prevents bacterial growth. Even though the American Association of Endodontists and the European Society of Endodontology recommend the use of $Ca(OH)_2$ as an intracanal medicament for regenerative endodontic procedures (134, 147), it should be known that $Ca(OH)_2$ provides no residual antimicrobial activity. This is important to consider when selecting an intracanal medicament for these procedures, as the goal is to create an aseptic environment allowing for continual root growth. Not only is it imperative to have a direct antibacterial effect, but with additional residual antimicrobial activity, an aseptic environment can be maintained for the initiation of root

development.

The AAE recommends utilizing an intracanal medicament such as DAP for a duration of 1 week to 4 weeks (134). Due to this suggestion, the current study investigated the residual antibacterial effect of radiopaque DAP at 1 week and 4 weeks. A previous study showed that 50 mg/mL and 500 mg/mL of DAP treatment for 1 week provided a significant and substantial residual bacterial effect (146). Another study exhibited residual antimicrobial activity for 2 weeks following treatment with 1 mg/mL of DAP for a three-week duration (42). This study presented that treatment time plays a significant role in disinfection. It was found that all tested concentrations of radiopaque DAP (1, 10, and 25 mg/mL) showed a significant reduction in bacterial count with 4 weeks of treatment when compared to 1 week of treatment. While it was shown that a longer treatment time rendered a more aseptic environment in all concentrations of DAP, it is still critical to consider the clinical manifestations and the severity of preoperative infection when determining an appropriate treatment time.

Several published studies have examined the cytotoxic effects of varying concentrations of DAP. It has been shown that an increasing concentration of antibiotic paste is associated with an increasing cytotoxicity to critical stem cells (32). Many of these studies have specifically investigated the deleterious effects of 1 mg/mL of DAP, which was the concentration used in the current study. Althumairy et al. demonstrated that the detrimental effects on SCAPs were largely prevented with concentrations of 1 mg/mL of DAP and lower (148). Furthermore, Ruparel et al. showed 1 mg/mL of DAP yielded a 56-perccent SCAP survival rate as opposed to 10 mg/mL leading to only a 20-percent viability of SCAPs (32). Labban et al. found that 1 mg/mL of DAP did cause

significant toxicity to human dental pulp cells when compared with an untreated control group (104). It seems clear that 1 mg/mL of DAP will lead to some degree of cytotoxicity to stem cells. With the incorporation of barium sulfate into DAP, it becomes imperative for future studies to evaluate the cytotoxic effects of varying concentrations of radiopaque DAP on dental stem cells.

SUMMARY AND CONCLUSIONS

My hypothesis, which stated that all tested concentrations (1 mg/mL, 10 mg/ML, and 25 mg/mL) of radiopaque DAP will have significant residual antibacterial effects, regardless of the application time, was accepted. In conclusion, this study suggested that all tested concentrations of radiopaque DAP were able to provide a significant residual antibacterial effect against bacterial biofilms isolated from an immature tooth with pulpal necrosis for both sets of treatment groups. Ca(OH)2 failed to provide any residual antibacterial effect once it was removed from the dentin samples.

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ABSTRACT

THE RESIDUAL ANTIBACTERIAL EFFECTS OF RADIOPAQUE DOUBLE ANTIBIOTIC PASTE AFTER VARIOUS TREATMENT TIMES

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Introduction: Regenerative endodontic procedures require adequate disinfection of immature teeth with necrotic pulps. Regeneration endodontic procedures have been shown to increase width and length of dentin after disinfection of the root canal system with various antibiotic pastes such as double antibiotic paste (DAP). DAP is composed of an equal ratio of metronidazole and ciprofloxacin. After the root canal system is disinfected with DAP, it is later flushed out with 17-percent EDTA. There is a need for a radiopaque component in the DAP to facilitate proper placement. Following disinfection with DAP, a residual antibacterial effect is formed in dentin. It is not known if this effect is modified by making DAP radiopaque. Objectives: The residual antibacterial effects of

a radiopaque double antibiotic paste against a bacterial isolate obtained from an immature tooth with necrotic pulp on radicular dentin will be explored utilizing various strengths of DAP (1 mg/mL, 10 mg/mL, and 25 mg/mL) after 1 week and 4 weeks. Materials and Methods: Dentin samples of 4x4-mm will be prepared from previously extracted human posterior teeth. The samples will be assigned to seven treatment groups in a random fashion (G1 will be treated with 25-, G2-10, and G3-1 mg/mL of radiopaque DAP. G4 will be treated with the radiopaque filler without DAP. G5 will be treated with $Ca(OH)_2$ and G6 sterile water with bacterial biofilm, and G7 will be sterile water). The groups will be treated for both 1 week 4 weeks. These samples will then be stored in a phosphate buffered saline (PBS) solution for 1 week and then inoculated with the cultured bacterial isolate from an immature tooth with necrotic pulp. The specimens will be incubated for three weeks to permit adequate formation of a biofilm. The biofilm will be detached, diluted and spirally plated onto blood agar plates and incubated for a total of 48 hours in 5-percent CO_2 at 37°C. The number of CFUs/mL will be counted using an automated colony counter. A two way ANOVA and Fisher's Protected Least Significant Differences test using 5-percent significance level will be used to evaluate the resulting data. Expected outcome: A residual antibacterial effect will be observed on radicular dentin samples when treated with radiopaque DAP. Conclusion: Using a radiopaque DAP that provides residual antibacterial effects, further awareness into regenerative endodontic procedures will be gained leading to alterations in treating such cases.

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